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Bovine herpesvirus type-1 glycoprotein K (gK) interacts with UL20 and is required for infectious virus production

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ABSTRACT

We have previously shown that the HSV-1 gK and UL20 proteins interact and function in virion envelopment, membrane fusion, and neuronal entry. Alignment of the predicted secondary structures of gKs encoded by BoHV-1, HSV-1, HSV-2, EHV-1 and VZV indicated a high degree of domain conservation. Two BoHV-1 gK-null mutant viruses were created by either gK gene deletion or stop codon insertion. In addition, a V5 epitope-tag was inserted at the carboxyl terminus of gK gene to detect gK. The engineered gK-null mutant viruses failed to replicate and produce viral plaques. Co-immunoprecipitation of gK and UL20 expressed via different methods revealed that gK and UL20 physically interacted in the presence or absence of other viral proteins. Confocal microscopy showed that gK and UL20 colocalized in infected cells. These results indicate that BoHV-1 gK and UL20 may function in a similar manner to other alphaherpesvirus orthologues specified by HSV-1, PRV and EHV-1.

1. Introduction

Bovine herpesvirus type 1 (BoHV-1) is a member of the alphaherpesvirus family and is the prototype herpesvirus of ruminants (Schwyzer and Ackermann, 1996). It is an important pathogen of cattle causing a wide variety of diseases including conjunctivitis, abortions, genital and reproductive disorders and upper respiratory tract infection also known as infectious bovine rhinotracheitis (IBR) (Biswas et al., 2013; Jones and Chowdhury, 2007; Tikoo et al., 1995). BoHV-1 is an important component of the Bovine Respiratory Disease Complex (BRDC) or "Shipping fever" that causes substantial economic losses to the US cattle industry annually (Ellis, 2009; Jones and Chowdhury, 2007, 2010). Based on genome analyses with restriction endonucleases and clinical observation, BoHV-1 is subdivided into three subtypes: BoHV-1.1 is associated with respiratory disease; BoHV-1.2a is most commonly linked to genital infections, and the less pathogenic BoHV-1.2b strains (Jones and Chowdhury, 2007, 2010).

The BoHV-1 genome consists of a linear double-stranded DNA molecule of about 136 kilobases (kb) long encoding 73 proteins (Schwyzer and Ackermann, 1996; Muylkens et al., 2007; Robinson et al., 2008). The genome is subdivided into unique long (UL) and unique short (US) segment with two inverted repeat sequences that flank the US region, the internal repeat (IR) and the terminal repeat (TR) regions (Muylkens et al., 2007; Schwyzer and Ackermann, 1996). The BoHV-1 genome is extremely guanine and cytosine rich (72%) and many proteins are conserved within the *Alphaherpesvirinae* subfamily (Muylkens et al., 2007). Like other alpha herpesviruses, BoHV-1 establishes life-long latency in ganglionic neurons after primary infection of the nasal epithelium (Jones, 2003, 1998). Reactivation and transmission of the virus to the other susceptible cattle occur after stress or corticosteroid-induced factors (Jones and Chowdhury, 2007).

Alphaherpesviruses encode several glycoproteins which are present in the viral envelope and play essential roles in viral pathogenicity and regulate virus attachment, entry, egress, and cell-to-cell spread. Like other alphaherpesvirus, BoHV-1 encodes two highly hydrophobic multiple membrane-spanning proteins, which are homologues of herpes simplex virus type 1 (HSV-1) glycoprotein K (gK) encoded by open reading frame 53 and a membrane protein encoded by UL20. It has been demonstrated for HSV-1, pseudorabies virus (PRV) and equine herpesvirus-1 (EHV-1) that these two proteins form a complex and that their co-expression is required for correct intracellular trafficking, localization and function (Dietz et al., 2000; Foster et al., 2008, 2004a; Guggemoos et al., 2006). The BoHV-1 gK is comprised of 338 amino acids with the apparent molecular mass of 36 kDa. gK is predicted to be a highly hydrophobic protein with four transmembrane domains, an N-terminal 29 aa cleavable signal sequence, and two N-glycosylation sites at asparagine positions 46 and 67 (Khadr et al., 1996). The BoHV-1 UL20 protein encodes 231 aa with the apparent molecular mass of 23 kDa. The UL20 protein is predicted to have four transmembrane domains without a signal sequence and any

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glycosylation sites. UL20 and gK orthologies encoded by HSV-1, PRV and EHV-1 have been shown to be essential for infectious virus production and virus spread (Chouljenko et al., 2010; Dietz et al., 2000; Foster et al., 2008; Foster and Kousoulas, 1999; Fuchs et al., 1997; Guggemoos et al., 2006; Jambunathan et al., 2014; Mo et al., 1999; Neubauer and Osterrieder, 2004).

Herein, we report that the BoHV-1 gK and UL20 proteins physically interact in the presence or absence of other viral proteins in a manner similar to the HSV-1 gK and UL20. In addition, we show that BoHV-1 gK is absolutely essential for infectious virus production and spread.

2. Materials and methods

2.1. Secondary structure modeling and Comparison

Amino acid sequence for BoHV-1 gK (GenBank: AAA84882.1), herpes simplex virus type −1 (HSV-1) gK (GenBank: ADD60046.1), HSV-2 gK (GenBank: AEV91393.1), Equine herpesvirus-1 (EHV-1) gK (GenBank: AII81659.1), and varicella zoster virus (VZV) gK (NCBI Reference Sequence: NP_040128.1) were uploaded to https://genesilico.pl/meta2 and annotated with the output metadata derived feature consensus. Graphical representation of feature locations conducted using Adobe Illustrator CS6.

2.2. Cells and viruses

Madin-Darby Bovine Kidney (MDBK) cells (ATCC CCL-22), and human embryonic kidney (HEK293) cells (ATCC CRL 1573) were propagated in Dulbecco's modified Eagle's medium supplemented with 5% and 10% fetal bovine serum respectively with 1% antibiotics. The cells were maintained at 37 °C in a 5% CO_2 and 95% air incubator. The BoHV-1 Cooper strain (ATCC VR-864) (a gift from Dr. Shafiq Chowdhury, Louisiana State University) was grown in MDBK cells and viral DNA was prepared (Inman et al., 2001; Saira et al., 2008) and used as template for all PCR-assisted cloning. The BoHV-1 BAC (Jura strain) has been described previously (Gabev et al., 2009) and was provided by Dr. Kurt Tobler at the Institute of Virology, University of Zurich, Switzerland.

2.3. BoHV-1 BAC mutagenesis

The BoHV-1 BAC plasmid (Jura strain) (Gabev et al., 2009) was used to generate the different gK mutant viruses. This BAC DNA was isolated from *E. coli* DH10B, transformed into E. coli GS1783 (a gift from Dr. Greg Smith, Northwestern School of Medicine, Chicago, IL), and used in two-step Red-mediated mutagenesis as described previously (Tischer et al., 2010, 2006) using 4 nmol ultramer primers synthesized by IDT (Table 1). Specifically, approximately100 ng of DpnI digested gel purified PCR product amplified from the pEPkan-S plasmid using the specific primers (Table 1) was transformed into the GS1783 competent cells with the Bio-Rad Pulser. The bacteria were cultured at 31 °C for 60 min and plated onto LB agar plates containing 30 μg/ml chloramphenicol and 30 μg/ml of kanamycin to select the kanamycin-resistance colonies. The selected clones were confirmed by PCR using the appropriate primers. The kanamycin-resistance gene was then excised by expressing I-SceI restriction enzyme, which was induced by adding arabinose to the culture medium followed by induction of the Red recombination system. Briefly, 100 μl of an overnight culture of the GS1783 cells containing the kanamycin-resistance gene was inoculated into 2 ml of LB media (1:20 ratio) containing 30 μg/ml chloramphenicol. The bacteria were cultured

Table 1
Primer sequences used in BoHV-1 BAC mutagenesis study.

Gene	Direction	Sequences
GFP-gK	Forward	*TAT* **aga tct** ATG CTG CTC GGG GGG CGG ACT
	Reverse	*TAT* **ggt acc** CGT CTG CGC GCC CAG CAG CCG
Flag-UL20	Forward	*AGA* **gga tcc** ATG CTC GGC CCG GAG AGT GCA
	Reverse	*ATA* **gtc gac** CAC GGC CAC GTG GCC CCC CAG
GST UL20/N	Forward	*AGA* **gga tcc** ATG CTC GGC CCG GAG AGT GCA
	Reverse	*ATA* **ctc gag** CGC GGC CCG CCC GTC CGC
gK del	Forward	CCC CAC CCT GCG CGG CAG CAG CGC CGC CCA ATA GGG GCG GAC CGC GCC GCG CGC CCC GAGAGG ATG ACG ACG ATA AGT AGG G
	Reverse	ACC GTG TTC ACC GTG GAT CTC TCG GGG CGC GCG GCG CGG TCC GCC CCT ATT GGG CGG CGC CAA CCA ATT AAC CAA TTC TGA TTA G
gK 34-35 S	Forward	CTG GCG CTG TGG GCC CCC CTC GCC GCG CCG CTG CGC TAG TGA TGT GCG CTC GCC GTC CGC GCC AGG ATG ACG ACG ATA AGT AGG G
	Reverse	GAG CGA GCC GTT GGC GCC GGT GGC GCG GAC GGC GAG CGC ACA TCA CTA GCG CAG CGG CGC GGC CAA CCA ATT AAC CAA TTC TGA TTA G
gK V5	Forward	TGT TCG CCG GCC CCA CCC TGC GCG GCA GCA GCG CCG CCC AAT AGG GGC GGC TAC GTA GAA TCG AGA CCG AGG AGA GGG TTA GGG ATA GGC TTA CCA GGA TGA CGA CGA TAA GTA GGG
	Reverse	TGC TGC ACT ACG AGC ACA ACC TGC GCC TGC GGC TGC TGG GCG CGC AGA CGG GTA AGC CTA TCC CTA ACC CTC TCC TCG GTC TCG ATT CTA CGT AGC AAC CAA TTA ACC AAT TCT GAT TAG
Kan PCR	Forward	AGG ATG ACG ACG ATA AGT AGG G
	Reverse	CAA CCA ATT AAC CAA TTC TGA TTA G
gK PCR	Forward	GCG TTT CTA AAA GAG GGC TC
	Reverse	GTA CCG TGT TCA CCG TGG AT

Lower case bold letters represent restriction endonucleases; sequences in upper case italics indicate additional bases added to restriction enzymes; red letters indicate insertion of nucleotides to introduce premature stop codons in gK gene.

for 2 h at 31 °C, and then 2% arabinose was added and cultured for another hour. The culture was transferred into a 42 °C water bath and incubated for half an hour and then transferred back to 31 °C shaker and continued for 1.5 h before being transferred to agar plates containing 30 μg/ml of chloramphenicol. Chloramphenicol-resistant, but kanamycin-sensitive clones were selected by plating single clones onto chloramphenicol- and chloramphenicol-kanamycin-containing plates.

2.4. BAC DNA isolation and analysis

BAC DNA was isolated by standard alkaline lysis from 5 ml of overnight bacterial cultures and the integrity of the BAC DNA was analyzed by digestion with restriction enzymes followed by separation of resultant DNA fragments by agarose gel electrophoresis. For confirmation of the engineered mutations, BAC DNA was amplified by PCR with specific primers and sequenced as well as analyzed by gel electrophoresis to exclude any unwanted mutations. For transfection and reconstitution of infectious virus, BAC DNA was isolated from bacteria harboring parental BoHV-1 and gK null viruses as by using a NucleoBond BAC 100 kit (Macherey-Nagel, Germany) as described earlier (Robinson et al., 2008) and following manufacture's instruction. Mutant BAC DNAs, as well as the genomes of infectious viruses recovered from BACs were sequenced and compared to the parental viral genome using Next Gen Sequencing on Ion-Torrent sequencer (Thermo-Scientific, Inc) at the LSU Core Laboratory GeneLab. There were no spurious mutations detected that caused amino acid changes.

2.5. Plaque morphology

Plaque morphology of wild-type and mutant gK mutant viruses was performed as described previously for (Lee et al., 2009; Meláncon et al., 2005). Briefly, MDBK cell monolayers were infected with wild-type and gK mutant viruses at 37 °C for 1 h and then overlaid with 0.6% carboxymethyl cellulose in DMEM containing 2% FCS. After incubation at 37 °C for 48 h, cells were fixed with ice-cold methanol and plaques were visualized after immunohistochemical staining with polyclonal anti-BoHV-1 rabbit sera.

2.6. Microscopy

Confluent monolayers of MDBK cells were grown on 4 well lab Lab-Tek Chamber slides (Sigma C6807) and infected with BoHV-1 gK V5 at a MOI of 0.001 at 4 °C. Infected cells were then incubated at 37 °C for 10 h. Cells were fixed and labeled with mouse anti-V5 (gK) and either polyclonal rabbit anti-BoHV-1 VP22 (1:1000) or polyclonal rabbit anti-UL20 (1:500). Secondary antibodies used were rat anti-mouse alexaflour 488 (1:1000) and goat anti-rabbit alexaflour 594 (1:1000). DAPI was used for nuclear staining. More than 100 individual infected cell foci were examined and representative pictures were selected for illustration purposes. Co-localization was analyzed using the Olympus FluoView software and statistics were generated by the frequency of emission events overlapping for the respective fluorophore indicated by Pearson's product-moment correlation coefficient. Correlation coefficients approximating +1 and greater than 0.75 indicate a significant positive correlation.

2.7. Expression plasmids

All primers used in generation of expression plasmids are shown in Table 1. The gK and UL20 genes encoded by the BoHV-1 (Cooper), Jura and K22 sequences were found to be identical (not shown). The BoHV-1 (Cooper) UL20 gene was amplified by PCR using gene-specific primers containing BamHI-SalI site and cloned into 3X FLAG-tagged expression vector pCMV-3Tag6 (Agilent technologies, CA). For cloning of gK gene, we followed the recently published sequences of gK (Khadr et al., 1996). Full-length gK sequence was also amplified with specific primers containing BglII-KpnI site and cloned into the green fluorescent protein (GFP)-tagged pEGFP-C1 (Clontech, CA). For protein expression, the UL20 and gK gene sequences were cloned into BamHI-XhoI site of an N-terminal HA-tagged (#88861) and GST-tagged (#88870) vectors (Thermo Scientific, IL) respectively. For prokaryotic expression, amino terminus fragment encoding 1–74 amino acids of UL20 was amplified with specific primers (Table 1) and cloned into the BamHI-XhoI site of pGEX- 4T-1 (GE Healthcare, NJ).

2.8. Preparation of the UL20 antiserum

The BoHV-1 UL20 gene product is predicted to possess four membrane spanning domains (Fig. 4A). To generate an antiserum against BoHV-1 UL20, the amino terminus hydrophilic regions spanning the amino acids 1–74 was fused to the glutathione *S*-transferase (GST) gene and the expressed protein was purified through affinity chromatography as described previously (Haque and Kousoulas, 2013). One rabbit was immunized four times at 3-week intervals by intramuscular injection of 200 μg of purified fusion protein emulsified in TiterMax Gold (Sigma T2684). Sera collected after immunization was purified using Nab™ antibody purification kits (Thermo Scientific, IL) according to the manufacturer's directions and used in specific experiments.

2.9. Co-immunoprecipitation of gK and UL20

HEK293 cells were transfected with 2 μg each of GFP-gK or Flag-UL20 expression plasmid in 6-well plate using either Genjet™ (SignaGen, MD) or the DNA Transfection Reagent (Biotool, TX), as recommended by the manufacturers. At 30 h post transfection, cells were washed with phosphate-buffered saline, lysed in lysis buffer (25 mM Tris-HCl, pH 8.00, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA) and used in the immunoprecipitation assays. Lysates were immunoprecipitated with the addition of either anti- GFP antibody (Genscript, NJ) or anti-Flag gel (Biotool, TX) and protein A/G resin (Santa Cruz Biotechnology, CA). The beads were washed four times in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and eluted in 30 μl 2X sample buffer and analyzed by Western blotting in conjunction with specific antibodies.

For co-immunoprecipitation of gK and UL20 expressed in BoHV-1 infected cells, MDBK cells were infected with wild-type and V5-tagged gK viruses at an MOI of 1. At 24 h post infection, cell lysates were prepared and immunoprecipitated with anti-UL20 Pab and V5 mab. BoHV-1 anti-VP22 Pab was included a control. Magnetic protein G beads were added to precipitate their targeted proteins. Beads was washed four time in TBS-T and bound proteins were eluted in 30 μl 2X sample buffer and analyzed by Western blotting with rabbit anti-BoHV-1 UL20 antibody. As an alternative approach, full-length BoHV-1 gK containing an N-terminal GST-tag and UL-20 gene encoding an N-terminal HA-tag were in vitro synthesized using a one-step human coupled IVT kit (Thermo Scientific, IL) as recommended by the manufacturer. Briefly, I μg DNA for each of GST vector and UL20 genes and 400 ng DNA for GST-gK gene were incubated with translation mix (#88881) either at 30 °C or 26 °C for 4–5 h. For binding study, 2 μl of GST and GST-gK IVT products were first immobilized to GST-Trap M (Chromotek, Germany) magnetic beads in GST-Tap dilution buffer at RT for 30 min. Unbound particles were washed away with TBS-T. Next, equal amounts of HA-tagged UL20 protein was added to each tube and incubated at RT for another 20 min. The beads were washed four times in TBS-T and eluted in 30 μl 2X sample buffer and analyzed by Western blotting in conjuction with specific

antibodies.

2.10. Western blot analysis

Cell lysates were prepared from transfected or infected cells at the indicated times in lysis buffer (25 mM Tris-HCl, pH 8.00, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA) supplemented with protease inhibitors and clarified by centrifugation at 15,000 rpm for 15 min at 4 °C. Cleared lysates were mixed with sample buffer and either heated at 92 °C for 5 min or kept at RT, separated through 4–20% express-plus gels (Genscript, NJ). Following electrophoresis, gels were transferred to nitrocellulose membranes using an iBlot transfer device (Invitrogen, CA) and blocked for 60 min in TBS-T containing 5% nonfat dry milk. Membranes were probed with the specific primary antibodies and then either with goat anti-mouse secondary antibody conjugated with HRP (Santa Cruz, CA) or Clean-blot™ IP detection reagent (HRP) (Thermo Scientific, IL). After washing the membranes 5–6 times with TBS-T, specific signal was detected either with Luminata™ Classico or Forte substrate (Millipore, MA) depending on the signal intensity of target proteins.

2.11. Next generation genomic DNA sequencing

DNA sequencing of the BoHV-1 Jura BAC and mutant viruses was performed using the Ion Torrent Personal Genome Machine (PGM) and the 316 sequencing Chip (Life Technologies). DNA preparations collected from overnight cultures of the bacteria containing the respective viral genome were extracted using the Qiagen Spin Mini Prep Kit (Qiagen). The Ion Xpress Plus Fragment Library Kit (Life Technologies) was used to prepare high-quality fragment libraries from approximately 1 μg of total DNA. Template-positive Ion Sphere Particles (ISPs) containing clonally amplified DNA were produced using the Ion OneTouch 200 Template Kit v2 DL (for 200 base-read libraries) with the Ion OneTouch instrument. The Ion OneTouch ES instrument was used to enrich ISPs intended for the Ion PGM System using the Ion PGM 200 Sequencing Kit.

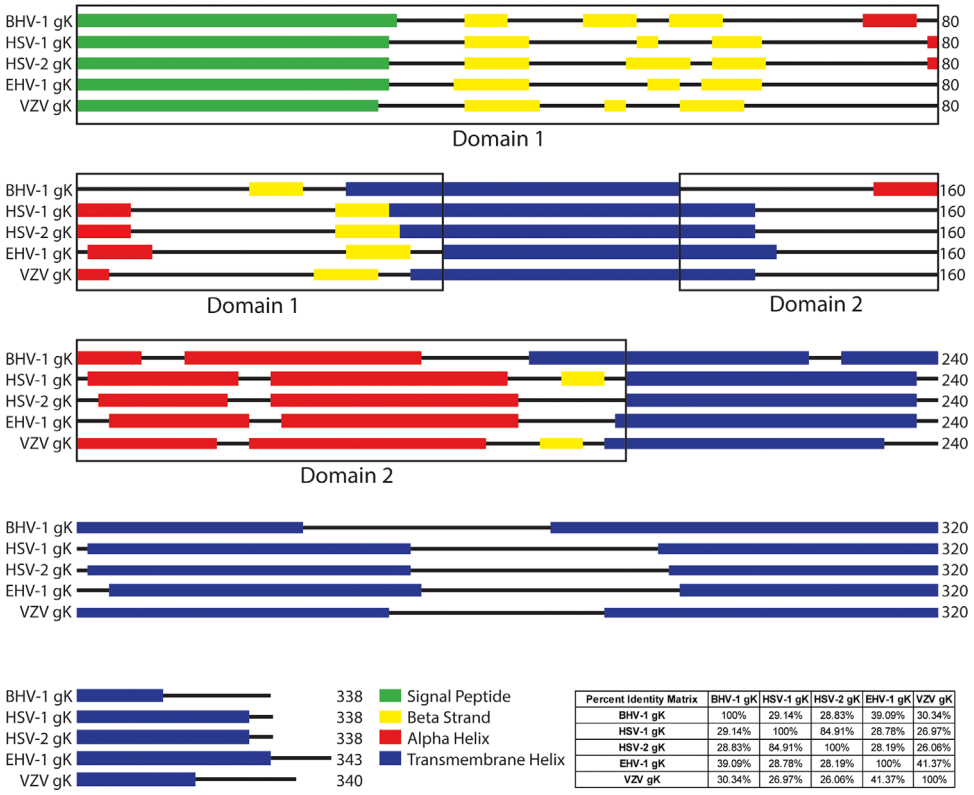
3. Results

3.1. Comparison of alphaherpesvirus gK secondary structures

Two-way comparisons of the primary sequences of gK encoded by BoHV-1, HSV-1, HSV-2, EHV-1 and VZV, revealed that BoHV-1 gK was more homologous to EHV-1 gK (39.09%-identity) and least homologous to the HSV-1 gK (29.14%) (Fig. 1). The secondary structures of gK encoded by these viruses were predicted using the Genesilico software, which utilizes multiple algorithms to arrive at a consensus secondary structure prediction, as described in Materials and Methods. Comparison of the predicted secondary structures revealed conservation of the signal and four transmembrane regions and similar predicted membrane topography with amino and carboxyl termini located extracellulary, as experimentally shown for the prototypic HSV-1 gK (Foster et al., 2003). Additional conserved domain features included an amino terminal region extending immediately after the signal sequence containing interspersed β-strands and a prominent predicted α-helical domain similar to our previously published HSV-1, HSV-2, VZV, and monkey B gK structures (Jambunathan et al., 2015) (Fig. 1).

3.2. Construction of gK mutant viruses

A set of recombinant viruses was constructed using two-step



Percent Identity Matrix	BHV-1 gK	HSV-1 gK	HSV-2 gK	EHV-1 gK	VZV gK
BHV-1 gK	100%	29.14%	28.83%	39.09%	30.34%
HSV-1 gK	29.14%	100%	84.91%	28.78%	26.97%
HSV-2 gK	28.83%	84.91%	100%	28.19%	26.06%
EHV-1 gK	39.09%	28.78%	28.19%	100%	41.37%
VZV gK	30.34%	26.97%	26.06%	41.37%	100%

Fig. 1. Alignment of gK predicted secondary structures encoded by alphaherpesviruses. Two-way amino acid sequence alignments were produced for BoHV-1, EHV-1, HSV-1, HSV-2 and VZV using the Clustal X software. Consensus metadata-derived secondary structures were generated using Genesilico (https://genesilico.pl/meta2) secondary structure prediction and aligned using MegAlign software. Essential features of gK secondary structures are color-coded as indicated.

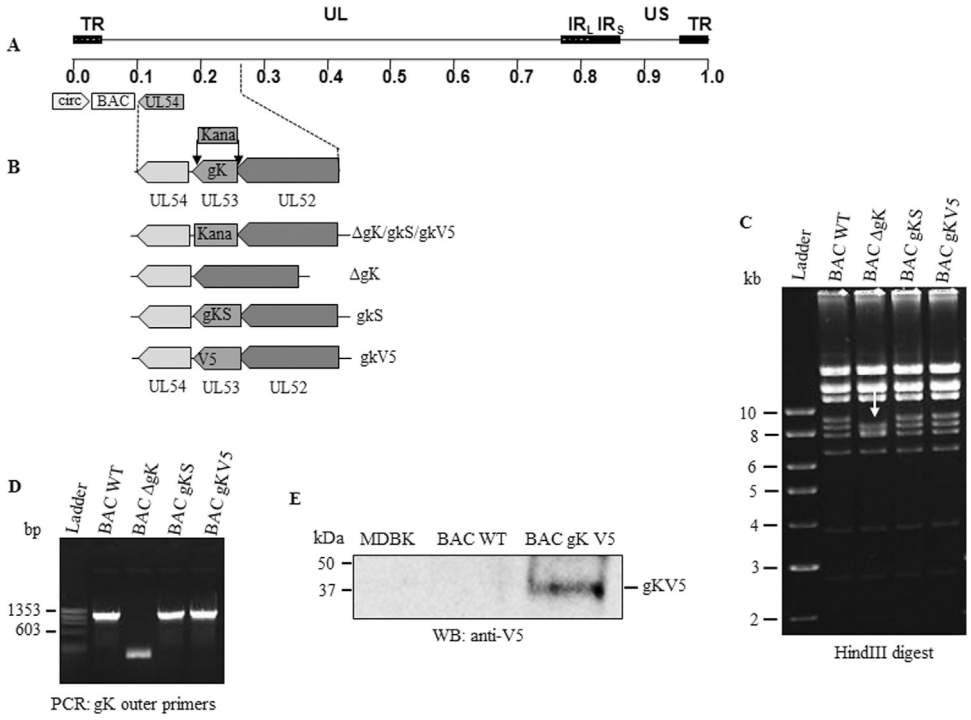


Fig. 2. Generation of BoHV-1 gK mutant viruses. Schematic showing the steps in generating gK mutant viruses. (A) The prototypic arrangement of the BoHV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. The BAC cassette was inserted between CIRC and UL54 genes of BoHV-1 Jura strain. (B) The genomic region of the UL53 (gK) flanked by the adjacent genes showing their orientation in the clusters. For construction of gK mutant viruses, either whole gK gene was deleted (ΔgK), or an in frame premature stop codons was inserted at aa 34–35 of gK gene (gKS). An in frameV5-tag was also inserted at C-terminus of gK gene for functional analysis (gKV5). (C) Identification of BoHV-1 mutant viruses by a combination of RFLP, PCR and Western blot analyses. BAC DNA from BoHV-1 parental as well as mutant viruses were digested with HindIII and analyzed by agarose gel electrophoresis. Fragment in the gK deleted virus that disappeared is indicated by an arrow. (D) Respective viral DNAs were analyzed by PCR with specific primers binding to the outside of the deleted or mutated gK gene. (E) Confirmation of V5-tag insertion to BoHV-1 gK gene by Western blot. MDBK cell lysates infected with wild-type and V5-tagged viruses were Western blotted and probed with mouse anti-V5 antibody.

Red-mediated recombination on the BoHV-1 (Jura) genome cloned as a bacterial artificial chromosome (BAC) (Gabev et al., 2009), as described in Materials and Methods. First, the gK-null mutant virus ΔgK was constructed by deleting the entire gK coding sequences (Fig. 2B). Next, the gK-null virus gKS was constructed by inserting two successive in-frame stop codons at gK amino acid positions 34–35 (Fig. 2B). Digestion of the wild-type BoHV-1 genome and the gKS and V5-tagged gK viruses with HindIII generated a fragment size of about 9 Kbp at the gK locus (Fig. 2C). Deletion of the gK gene resulted in a change in restriction pattern of the ΔgK virus causing the disappearance of this 9 Kbp band. The gK gene deletion is approximately 1 kbp. Therefore, a new band was expected to appear with an apparent molecular mass of approximately 8 kbp. As evidenced in the restriction profile, the 8 kbp band is denser indicating the presence of this deleted DNA fragment most likely, originating from the original 9 kbp DNA fragment (Fig. 2C). In addition, PCR amplification of the targeted genomic region with primers binding to the outside of the gK gene confirmed the deletion of the gK gene (Fig. 2D). The gKS stop codon mutations were confirmed by sequencing PCR amplified fragments spanning these mutations (not shown). To facilitate detection of the BoHV-1 gK gene, a V5 epitope tag was inserted in-frame at the carboxyl terminus of gK generating mutant virus gKV5 and confirmed by PCR-assisted nucleotide sequencing and Western immunoblot analysis of cellular extracts obtained from MDBK cells infected with the gKV5 virus revealing gK migrating with an apparent molecular mass of approximately 37 kDa (Fig. 2E). Side-by-side Next Generation Sequencing of the entire viral genomes of the BoHV-1 (Jura) BAC and the derived mutant viruses gKS, ΔgK and gKV5, revealed that there were no spurious mutations coding for any amino acid changes (not shown).

3.3. Plaque morphologies of BoHV-1 mutant viruses

To examine whether infectious virus can be recovered from the mutant BAC constructs, purified BAC genomic DNAs were transfected into HEK293 cells using the ViaFect transfection reagent (Promega, WI). HEK293 cells were used because of their higher transfection efficiency as demonstrated in the recovery of the BAC-cloned EHV-4 genome (Azab et al., 2010a, 2010b). The parental wild-type BAC genome produced a number of viral plaques visualized by fluorescence microscopy, since the virus constitutively expresses the green fluorescence protein (GFP) gene inserted within the BAC, as well as ample evidence of cytopathic effects. In contrast, both ΔgK and gKS BAC-transfected cells revealed the presence of single fluorescent cells and lack of any observable cytopathic effects (Fig. 3A and B). HEK293 cells transfected with each of the wild-type, ΔgK and gKS BACs were collected and virus stocks were prepared to test for the presence of infectious virions. Infection of MDBK cells with these virus stocks and detection of viral infection by immunohistochemistry using anti-BoHV-1 polyclonal antibody revealed that only the wt BAC produced viral plaques, while both ΔgK and gKS virus stocks produced a low number of single cells stained with the antibody indicating the inability of these viruses to spread (Fig. 3C).

3.4. Detection of the BoHV-1 UL20 protein using the anti-UL20-specific antibody

The BoHV-1 UL20 protein is predicted to have four transmembrane domains (Fig. 4A). To identify and characterize this protein, the amino-terminal hydrophilic region spanning amino acids (aa) 1–74 of the UL20 protein was expressed as a GST fusion

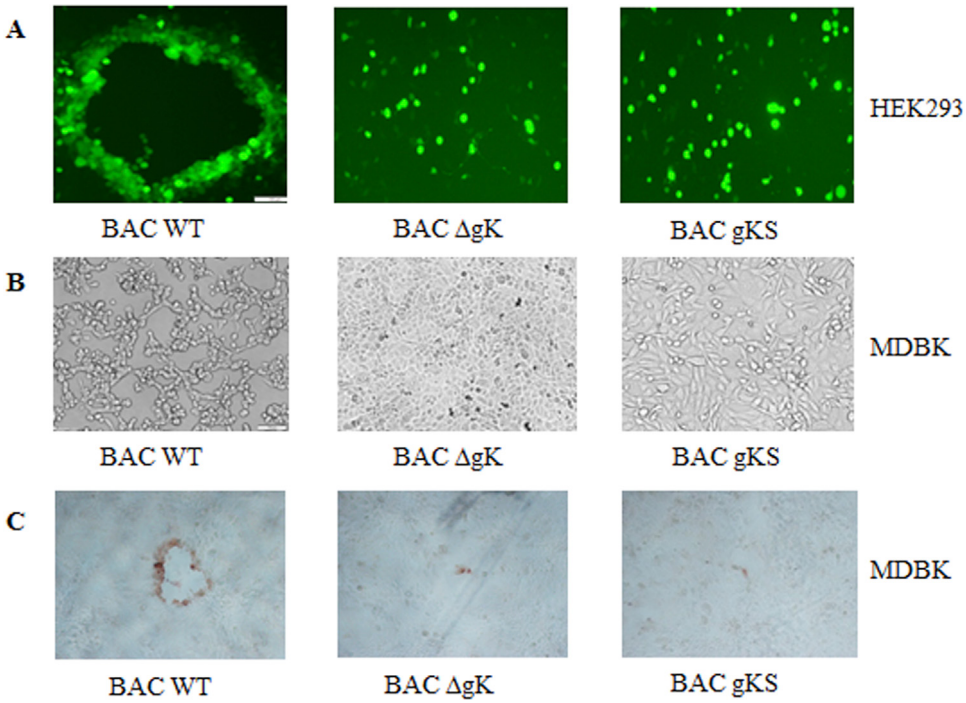


Fig. 3. BoHV-1 BAC virus reconstitution. (A) DNA transfected to HEK293 cells produced plaques by wild-type virus, but not by gK mutant viruses (Upper panel). After 3 days post-transfection, specific viruses were recovered and infected to MDBK cells. (B) Wild-type virus caused cytopathic effects (CPE), while no CPE was observed in gK mutant viruses (middle panel). (C) MDBK cell monolayers were infected with wild-type and gK mutant viruses at 37 °C for 1 h and then overlaid with 0.6% carboxymethyl cellulose in DMEM containing 2% FBS and incubated at 37 °C for 48 h. Cells were then fixed with ice-cold methanol and plaques were visualized after immunohistochemical staining with a polyclonal anti-BoHV-1 rabbit sera (lower panel).

protein in *E.coli*. This fusion protein was purified using glutathione sepharose and its purity was assessed by SDS-PAGE and Coomassie blue staining (Fig. 4B). One rabbit was immunized with this purified antigen to generate antiserum. By Western blot analysis, this antiserum recognized a single band of a 23-kDa protein in BoHV-1 infected MDBK cell lysates, but not in mock-infected cell lysates (Fig. 4C). Preimmune control serum did not react with this protein species (data not shown). In addition, the anti-UL20 serum was utilized to detect UL20 expressed by in vitro-transcription-translation. The anti-UL20 serum reacted with both wt UL20 and UL20 tagged with the FLAG epitope inserted at the amino-terminus of UL20 (Fig. 4D).

3.5. Interaction of gK with UL20 in BoHV-1 infected cells

We have previously shown that the HSV-1 gK and UL20 proteins interact and function as a functional complex in regulating cytoplasmic virion envelopment, gB induced cell fusion, and required for neuronal entry (David et al., 2012; Foster et al., 2008, 2001). To test potential interaction between BoHV-1 gK and UL20 proteins, a coimmunoprecipitation assay was performed using proteins expressed BoHV-1 infected cells. MDBK cell lysates were prepared from BoHV-1 wild-type and gKV5 viruses at 24 hpi and immunoprecipitated with anti-VP22 antibody (negative control), anti-UL20 antibody (positive control) and anti-V5 monoclonal antibody (positive control for the V5 tag). The immunoprecipitates

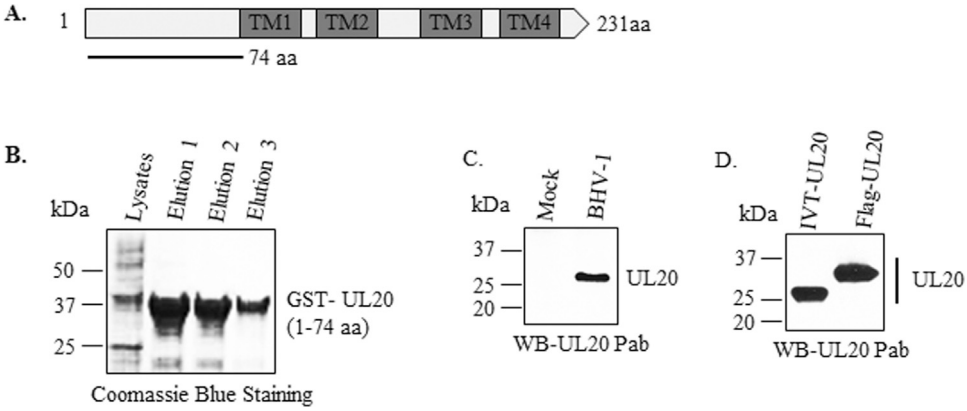


Fig. 4. Membrane topology and generation of UL20 anti-serum. BoHV-1 UL20 protein encodes 231 amino acids and has four transmembrane domains (A). To generate UL20 anti-serum, N-terminal fragment encoding amino acids 1–74 was fused in frame with GST and purified through affinity chromatography. (B) Purity of the GST-UL20 protein as assessed by SDS-PAGE and Coomassie blue staining. (C&D) Specificity of UL20 anti-serum by Western blot. (C) Lysates from mock or BoHV-1 infected MDBK cells 40 h post-infection. (D) UL20 protein was either in vitro translated or expressed as Flag-tagged to HEK293 cells. Respective proteins were separated through SDS-PAGE and subjected to Western blotting using anti-UL20 anti-serum.

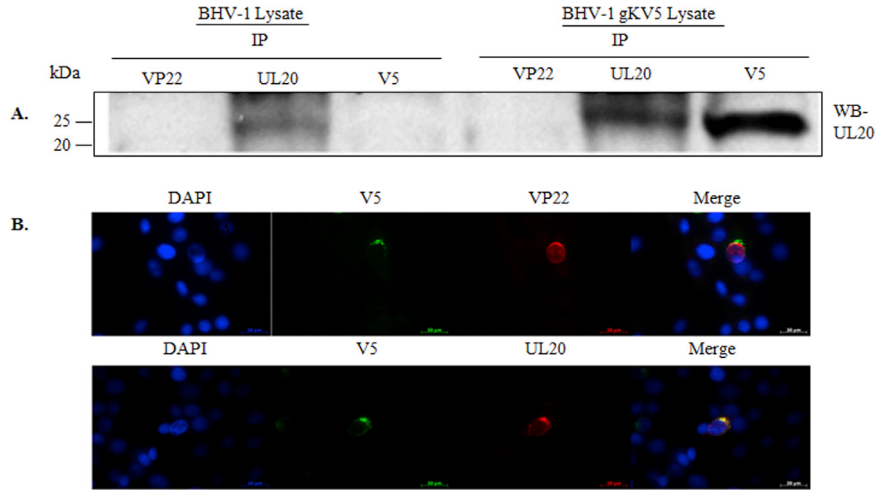


Fig. 5. Co-immunoprecipitation and colocalization of BoHV-1 gK with UL20. (A) Cell lysates were prepared from BoHV-1 wild-type and gK V5-tagged infected MDBK cells at 24 h post infection. Anti-BoHV-1 VP22, UL20 rabbit antibodies and mouse-derived anti-V5 antibody were used with magnetic protein G beads to precipitate their targeted proteins from infected cell lysates. Membranes were probed with polyclonal rabbit anti-BoHV-1 UL20. (B) Co-localization of BoHV-1 gK with UL20. MDBK cells were infected with BoHV-1 gK V5 at a MOI of 0.001 and incubated at 37 °C for 10 h. Cells were fixed and labeled with mouse anti-V5 (gK) and either polyclonal rabbit anti BoHV-1 VP22 (top) or polyclonal rabbit anti UL20 (bottom). Secondary antibodies used were rat anti-mouse alexaflour 488 and goat anti-rabbit alexaflour 594. DAPI was used for nuclear staining. Infected cells shown are representative staining of more than 100 infected cell foci that were examined with similar staining patterns.

were probed with anti-UL20 antibody by Western immunoblotting to detect the specific interaction of UL20 with gK gene. The anti-UL20 antibody detected UL20 migrating with the expected molecular mass of 23 kDa in gKV5 infected cell lysates, but not from wild-type virus-infected cell lysates, or from lysates reacted with the anti-VP22 antibody (Fig. 5A). Next, to explore if gK and UL20 proteins co-localize within the BoHV-1 infected cells, MDBK cells were infected with the gKV5 virus, fixed and processed for confocal microscopy using antibodies against V5(gK), VP22 and UL20, as described in Materials and Methods. Colocalization signals were detected for gK and UL20 in infected cells using the anti-UL20 antibody, but not the VP22 antibody (Fig. 5B). Computational analysis of gK and UL20 colocalization signals showed a significant positive relationship between the location of their respective signal emissions (Pearson's Correlation Coefficient=0.92846) (Fig. S1).

3.6. Confirmation of gK-UL20 interactions

To further validate gK-UL20 interactions, we tested whether the BoHV-1 gK can physically interact with UL20 protein in transfected cells. For this purpose, gK and UL20 genes were cloned into a GFP- and Flag-tagged expression vector and used in cotransfection experiments. HEK293 cells were transfected with GFP-gK alone, or in the presence of the Flag-UL20 plasmid, and the parental control plasmid vector was used to normalize the total DNA. At 30 h post transfection, cell lysates were prepared and first immunoprecipitated with anti-GFP polyclonal antibody and then immunoblotted with anti-Flag monoclonal antibody to detect the presence of Flag-UL20 bound to GFP-gK. The anti-GFP antibody coimmunoprecipitated Flag-UL20 from the cell extract obtained from cells cotransfected with Flag-UL20 plasmid, but not from the cell lysates transfected with control vector (Fig. 6, left panel). In the reverse experiment, the anti-Flag resin coimmunoprecipitated GFP-gK only in the presence of Flag-UL20, but not from control cell lysates (Fig. 6A, right panel). The presence of GFP-gK and Flag-UL20 proteins in the cell extracts was detected with their respective antibodies (Fig. 6A bottom panel). Next, to further confirm the above observed gK-UL20 interactions, gK and UL20 were expressed by in vitro transcription-translation. Both full-length gK and UL20 proteins were expressed as N-terminal fusions to GST and HA tags, respectively, and used in binding experiments as described in Materials and Methods. The relative expression of GST and GST-gK proteins used in these binding assays was ascertained by immunoblot detection of 10% of the input samples using anti-GST antibody (Fig. 6B). These experiments, revealed that the HA-UL20 specifically bound to GST-gK, but not to the control GST vector (Fig. 6C). Collectively, these results demonstrate that BoHV-1 gK and UL20 proteins physically interact in the presence and absence of other viral proteins.

4. Discussion

Herein, we demonstrate that predicted secondary structures of gK encoded by different alphaherpeviruses are conserved suggesting these specific gK domains may serve similar functions. We have previously shown that the HSV-1 gK and UL20 form a functional protein complex that is necessary for their inter-dependent functions in virus-induced cell fusion, virus entry, infectious virus production and spread. We constructed BoHV-1 gK null viruses and found that BoHV-1 gK is essential for infectious virus production and spread. In addition, we found that BoHV-1 gK physically interacts and colocalizes with UL20 in infected cells. These results suggest that BoHV-1 gK and UL20 may serve similar functions to those described for the prototypic HSV-1.

Metadata-derived consensus secondary structure prediction of gK encoded by BoHV-1, HSV-1, HSV-2, EHV-1 and VZV viruses revealed surprising conservation of extracellular and intracellular domains including an ecto-domain containing β-strands and a prominent α-helical cytoplasmic domain. Also, we have previously shown that deletion of 38 amino acids from the amino terminus of the HSV-1 gK prevents viral entry into the axonal compartments of neurons (Jambunathan et al., 2015). This HSV-1 domain contains two N-linked glycosylation sites that are spatially conserved among most alphaherpesviruses including BoHV-1 gK. This conservation suggests that this amino terminal domain may serve similar functions for BoHV-1 gK, as it has already been shown for HSV-1 gK.

Disruption of HSV-1 gK results in significantly reduced plaque size and infectious virus production (David et al., 2008, 2012; Foster and Kousoulas, 1999; Foster et al., 2001; Iyer et al., 2013; Jayachandra et al., 1997), however a greater defect has been reported for VZV (subfamily varicella virus) (Mo et al., 1999)

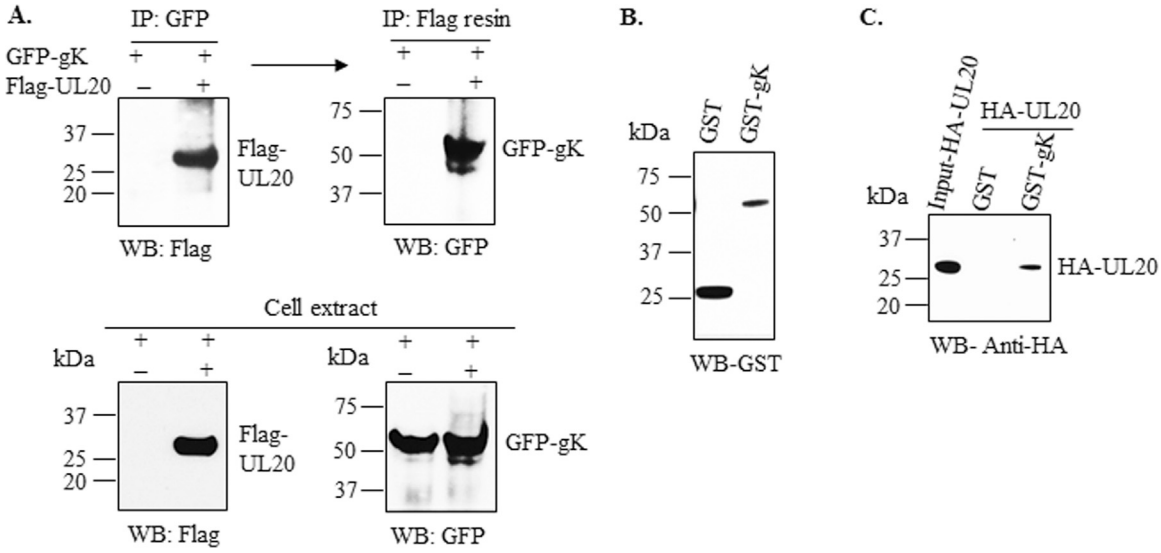


Fig. 6. Interactions of gK and UL20. (A) Two-way co-immunoprecipitations of BoHV-1 gK and UL20 proteins. HEK 293 cells were transiently transfected with GFP-tagged gK or co-transfected with Flag-tagged UL20 protein, as indicated combination. Whole cell extracts were immunoprecipitated (IP) using either anti-GFP antibody or anti- FLAG gel. The immunoprecipitates were analyzed by Western blotting with specific antibodies. The presence of Flag-UL20 and GFP-gK in the cell extracts were analyzed by Western blotting with specific antibodies. (B) Full-length BoHV-1 gK was expressed as GST- fusion protein using Thermo Scientific in vitro translation systems and expression was verified using 10% of input protein used in binding by Western blotting with anti-GST antibody. (C) In vitro interactions of gK and UL20. Equal amounts of HA-tagged in vitro synthesized UL20 was incubated with GST and GST-gK immobilized to GST-Trap agarose and specific interactions were detected by Western blotting with anti-HA mAb.

requiring complementation for plaques larger than one or two cells, which is similar to our findings with BoHV-1. Deletion of the entire BoHV-1 gK open reading frame (ΔgK) or insertion of consecutive premature stop codon at amino acids 34–35 of the gK gene (gKS) resulted in a severe virus spread defects, as evidenced by the inability of these viruses to form viral plaques and spreading infection beyond a single infected cells. This virus plaque phenotype is substantially more severe that observed in HSV-gK null viruses, which produce small viral plaques containing 10–20 infected cells suggesting that the BoHV-1 gK is essential for BoHV-1 infectious virus production and spread. Furthermore, co-immunoprecipitation and co-localization assays revealed that BoHV-1 gK and UL20 interact in infected cells, as well in the absence of other viral proteins in agreement with results obtained with the HSV-1 gK and UL20.

All alphaherpesviruses encode highly conserved gK and UL20 proteins suggesting that they may serve similar functions in membrane fusion, virion envelopment, and virus egress and spread (Dietz et al., 2000; Foster et al., 2008, 2004b; Guggemoos et al., 2006; Melancon et al., 2007). Delineation of the functional domains of BoHV-1 gK may aid in the development of vaccines and therapeutics for the prevention of widely distributed diseases. In support of this hypothesis, we have already demonstrated that a mutant HSV-1 carrying mutations in gK that prevented the virus from entering into neuronal axona, was highly effective as a vaccine in ameliorating genital herpes infections with strain independent virulent HSV-1 and HSV-2 (Iyer et al., 2013; Stanfield and Kousoulas, 2015; Stanfield et al., 2014). It is highly likely, that a similar live-attenuated BoHV-1 strain may be produced as an effective vaccine for preventing IBR in cattle, as well as a vector strategy for heterologous vaccine production against other major animal pathogens.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.09.003.

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